DOCKET NO. 3802-126-27 CONT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF:	De-Chao YU, et al.	Art Unit:	1633
SERIAL NO.:	10/691,045	Examiner:	Maria Marvich
FILING DATE:	October 21, 2003		
FOR:	CELL-SPECIFIC ADENOVIRUS VECTORS COMPRISING AN INTERNAL RIBOSOME ENTRY SITE		

AFFIDAVIT OF PRIOR INVENTION UNDER 37 CFR 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, DE-CHAO YU, being duly sworn, depose and state:

1. This Declaration is submitted as evidence that the subject matter claimed in the above-identified application was reduced to practice by the present inventors prior to the publication of Chang et al (WO 99/25860).

2. I understand that the Patent Application was filed on October 21, 2003 with the United States Patent & Trademark Office by Piper Rudnick, and was accorded Serial No. 10/691,045.

3. Andrew Little and I are co-applicants of the above-identified patent application, and the inventors of the subject matter described and claimed therein.

4. Throughout the time period from 1999 to 2001, I was Vice President of Research at Calydon Corporation and was involved in and supervised all projects directed to development of adenovirus vectors, more specifically, Attenuated Replication-Competent Adenovirus ("ARCA") vectors for local and systemic treatment of cancer. One project

of cancer. One project that I supervised was specifically directed to development of ARCA vectors comprising an internal ribosome entry site or "IRES".

5. In my role as Vice President of Research at Calydon Corporation, I supervised the work of Andrew Little who supervised the technical activities performed by Melissa Scott on the project directed to development of ARCA vectors comprising an internal ribosome entry site or "IRES".

6. This Declaration is submitted as evidence that the subject matter claimed in the above-identified application was reduced to practice by the inventors of the instant application prior to the publication of Chang et al. as explained below.

7. I have reviewed the presently pending claims numbered 59-88, as set forth in the amendments dated June 15, 2005 and October 12, 2005.

8. Prior to the publication of Chang et al (WO 99/25860; published May 27, 1999), the inventors of the above-identified application conceived the invention as claimed in at least Claims 59-88 of U.S. Application Serial No. 10/691,045, as noted herein with respect to Exhibits 1-8, which are all dated prior to May 27, 1999. Note that the dates have been redacted from Exhibits 1-8.

9. Page 70-71 of Cell Genesys Notebook # 96 issued to Andrew Little, provides a copy of a memo to me, "DC" from Andrew Little "Andy" (Exhibit 1). The subject matter of the memo is "IRES Strategy" and it is dated December 31, 1998. The memo describes the IRES strategy that was implemented in the experiments summarized in the subsequent pages of notebooks issued to Andrew Little and Melissa Scott. The memo states that the purpose of the project is to regulate expression of both the E1a and E1b genes using a single tissue specific promoter. Both the ECMV and VEGF IRES are referred to in the memo.

10. The memo makes reference to a strategy for cloning a platform plasmid for constructing Ad mutants that express IRESs. The cloning strategy is summarized in a hand written diagram found on page 72 of Genesys Notebook # 96 issued to Andrew Little (Exhibit 2).

11. The experimental details for cloning the platform plasmid and constructing Ad mutants that express IRESs are found on pages 73-117 of Cell Genesys Notebook # 96 issued to Andrew Little (Exhibit 3). The notebook pages describe the construction of two vectors in which the expression of the adenoviral E1a gene is under operative control of either a probasin or CMV promoter and expression of the adenoviral E1b gene is under operative control of an ECMV IRES.

12. Page 117 of Cell Genesys Notebook # 96 states "cloning turned over to Melissa Scott". (Exhibit 3).

13. Page 114 of Cell Genesys Notebook # 99, issued to Melissa Scott states that the goal of the experiment is to regulate expression of both the E1A and the E1B gene using a single tissue specific promoter and that to do so an internal ribosome entry site or (IRES, an internal entry point for initiation of translation by eukaryotic ribosomes) must be cloned between the 2 genes. Both the EMCV and VEG-F IRESs are described. Page 114 further states "Picking up project in the beginning.....where Andy left off" (Exhibit 4).

14. Pages 115-119 of Cell Genesys Notebook # 99, summarize the construction of an adenoviral platform plasmid which has the E1A and E1B genes separated by an EMCV IRES and use of the platform plasmid to make adenoviral vectors which include two different promoters, the probasin ("PB") or cytomegalovirus ("CMV") promoter (Exhibit 5). The experiments detailed by Melissa on pages 116-119 confirm the presence of the IRES in two clones prepared by Andrew Little.

15. The experiments detailed by Melissa Scott on pages 119 and 121 of Cell Genesys Notebook # 99 (Exhibit 6), describe addition of a CMV promoter to the adenoviral platform plasmid. The experiments detailed by Melissa on page 120 and describe addition of a probasin promoter to the adenoviral platform plasmid. (Exhibit 6).

16. Pages 133-134 of Cell Genesys Notebook # 99, issued to Melissa Scott summarize the analysis of constructs and confirmation of the insertion of the CMV and probasin

promoters (Exhibit 7). Five out of eight constructs were demonstrated to have the promoter in the correct orientation (page ***; Exhibit 8).

17. It should be noted that any date blocked out in Exhibits 1-8 were prior to the publication date of the Chang et al. (WO 99/25860), published May 27, 1999.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed at San Francisco, California, this 2nd day of May, 2006

DE-CHAO YU

STATE OF California :

COUNTY OF San Francisco:

Before me, a Notary Public for said County, personally appeared DE-CHAO YU, known to me to be the person who executed the foregoing affidavit and acknowledged it to be his act and deed.

Witness my hand and seal this 2nd day of May, 2006

T. Moore

TA MOORE
COMM. #1638748
NOTARY PUBLIC-CALIFORNIA
SAN FRANCISCO COUNTY
My Commission Expires
JANUARY 17, 2010

CALYDON

TO: DC
FROM: ANDY
SUBJECT: IRES STRATEGY
DATE:
CC:

INTRODUCTION

The purpose of the project is to regulate the expression of both the E1a gene and the E1b gene using a single tissue specific promoter. One approach is to engineer both genes to be expressed in one mRNA. To insure efficient translation of the second cistron, an internal ribosome entry site will be cloned between the two genes. After reviewing relevant literature, I determined that two IRESes may be suitable. The first IRES is from encephelomycarditis virus (EMCV). The EMCV IRES has been well characterized. Researches have delineated the essential bases, and it is commercially available from Novagen (Madison, WI). Others have used the EMCV IRES in gene therapy studies using replication defective retroviruses and adenoviruses. The second IRES selected is from the mouse VEG-F gene. Several genes that regulate cell proliferation and promote cell transformation, like fibroblast growth factor and insulin growth factor, contain IRESes. One hypothesis for why cellular proliferation genes contain IRESes is that cap-dependent translation is not efficient under hypoxic conditions. Huez et al. (1998) recently described a truncated 163 base IRES from a differentially spliced VEG-F mRNA. Their work showed that this IRES was more active than the full length VEG-F IRES and had activity in human cells. A human VEG-F IRES was also recently described. However, it has two independent elements that total 700 bases.

PLATFORM PLASMID CONSTRUCTION

I have attached a strategy for cloning a platform plasmid for constructing Ad mutants that contain IRESes. The first step is to create a deletion in CN306, a plasmid derived from pXC.1 lacking the E1a promoter, that removes the E1B promoter and E1a poly A signal, but leaves the E1a and E1b coding regions intact. This can be accomplished by overlap PCR (see attached). Primers A and C amplify a 288 bp fragment, and primers B and D amplify a 396 bp fragment. Primers C and D were designed to contain 15 b of homology to each other so that the two PCR products could be annealed. This 15 bp region of homology also contains a unique Sal I site that will be

Witnessed & Understood by me,

Christa M. Mel

Invented by

Recorded by

used to clone in the IRESes. The Sal I site has been engineered so that IRES inserts will be placed 6 bases (or 2 amino acids) upstream of the E1b translation initiation codon. The overlap product (669 bp) will then be digested with Xba I and Kpn I and cloned into similarly cut CN306. All together, these manipulations delete about 118 bp of endogenous Ad sequence. Once the platform plasmid has been engineered, viruses can be generated by homologous recombination with Ad right hand end plasmids in 293 cells.

EMCV IRES

The EMCV IRES can be cloned from a commercially available plasmid (pCITE-3) using PCR. I suggest using pCITE-3 as a template because the IRES has been modified for optimal performance. Primers E and F amplify a 519 bp IRES fragment with Sal I ends that can be ligated into the platform plasmid described above. The EMCV IRES contains a pyrimidine rich tract of bases near its 3' end that are important for its activity. This pyrimidine tract will be 27 bases (9 amino acids) from the E1b initiation codon.

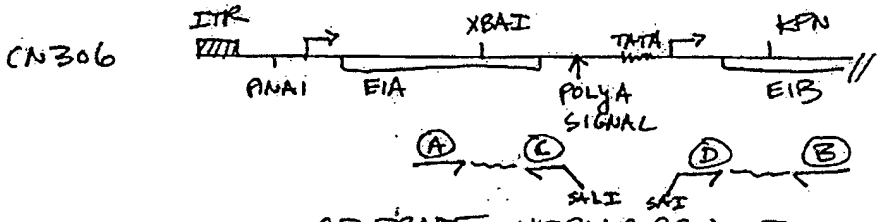
VEG-F IRES

Since the VEG-F IRES is only 163 bases, it may be possible to synthesize it with Sal I linkers. Because this IRES is from an alternatively spliced mRNA and cloning it would require several steps, synthesizing it may save us valuable time. The proposed sequence is attached. Once synthesized, the IRES could be easily cloned into the platform described above.

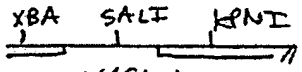
INTELLECTUAL PROPERTY ISSUES

One issue that needs to be considered before beginning the project is if patents applications have been filed on any of these IRESes. If so, do we need to license the IRESes? Or, do we proceed anyway to assess project feasibility before negotiating licensing terms? I suggest that we do a comprehensive intellectual property search.

PLATFORM PLASMID CONSTRUCTION



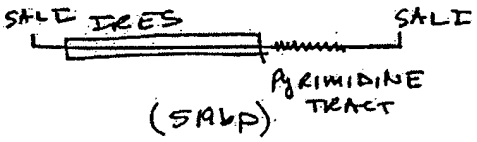
GENERATE OVERLAP PRODUCT TO CREATE DELETION OF E1B PROMOTER ETC.



(669bp)

OVERLAP PRODUCT CAN BE CLONED DIRECTLY BACK INTO CN306 TO CREATE DESIRED PLATFORM PLASMID WITH UNIQUE SALI SITE.

EMCV IRES



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VEGF IRES

SENSE PRIMER.

5' ACGTA SALI GTCGAC AGCGCAGAGGCTTGGGGCAGCCGAGCGGCAGCCA

GGCCCCGGCCCGGGCCTCGGTTCCAGAAGGGAGAGGAGCCCGCCAAGGCG

CGCAAGAGAGCGGGCTGCCTCGCAGTCCGAGCCGGAGAGGGAGCGCGAGC 1856

CGCGCCGGCCCCGGACGGCCTCCGAAACC ATG SALI GTCGAC ACGTA 3'

START CODON

ANTISENSE PRIMER

5' TACGT SALI GTCGACCATGGTTTCGGAGGCCGTCCGGGGCCGGCGCGGCTCGCG

CTCCCTCTCCGGCTCGGACTGCGAGGCAGCCCGCTCTCTTGCGCGCCTTGG 1851

CGGGCTCCTCTCCCTTCTGGAACCGAGGCCCGGGCCGGGGCCTGGCTGCCG

CTCGGCTGCCCCAAGCCTCTGCGCTGTCGAC SALI TACGT 3'

THESE TWO OLIGS WILL BE ANNEALED AND CLONED INTO UNIQUE SALI SITE OF PLATFORM PLASMID.

NOTE - AFTER DISCUSSION WITH KEYSTONE, I WILL DIVIDE EACH ABOVE OLIGO IN TWO AND SYNTHESIZE ~~2~~ 4 ~906 OLIGOS INSTEAD OF 2 ~1806 OLIGOS. APPARENTLY, LONGER OLIGOS CAN CONTAIN DELETIONS/INSERTIONS.

Witnessed & Understood by me.

Invented by

IRES CLONING OLIGOS

Amplification of EMCV IRES from Novagen pCITE-3a(+) vector

Sense

(96.74.1) GAC GTC GAC ATC GTG TTT TTC AAA GGA A (pCITE sequence 9 to 27) 28

Antisense

(2) GAC GTC GAC TAA TTC CGG TTA TTT TCC A (pCITE sequence 491 to 509) 28

Amplification of E1a/E1b intergenic region

Sense

Primer A
(3) CCT GAG ACG CCC GAC ATC ACC TGT G (Ad sequence 1314 to 1338) 25

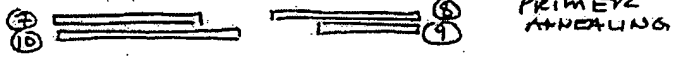
Primer D
(4) TGC TGA ATG GTC GAC ATG GAG GCT TGG GAG (Ad sequence 1714 to 1728) 30

Antisense

Primer B
(5) CAC AAC CGC TCT CCA CAG ATG CAT G (Ad sequence 2070 to 2094) 25

Primer C
(6) GTC GAC CAT TCA GCA AAC AAA GGC GTT AAC (Ad sequence 1572 to 1586) 30

Mouse VEG-F oligos to reconstruct SP163

Sense primers 

(7) ACG TAG TCG ACA GCG CAG AGG CTT GGG GCA GCC GAG CGG CAG CCA GGC CCC GGC CCG GGC CTC GGT TCC AGA AGG GAG AGG AGC CCG CCA 90mer

(8) AGG CGC GCA AGA GAG CGG GCT GCC TCG CAG TCC GAG CCG GAG AGG GAG CGC GAG CCG CGC CGG CCC CGG ACG GCC TCC GAA ACC ATG GTC GAC ACG TA 98mer

Antisense primers

(9) TAC GTG TCG ACC ATG GTT TCG GAG GCC GTC CGG GGC CGG CGC GGC TCG CGC TCC CTC TCC GGC TCG GAC TGC GAG GCA GCC CGC TCT CTT GCG C 94mer

(10) GCC TTG GCG GGC TCC TCT CCC TTC TGG AAC CGA GGC CCG GGC CGG GGC CTG GCT GCC GCT CGG CTG CCC CAA GCC TCT GCG CTG TCG ACT ACG T 94mer

notes – underlined regions indicate homology used for overlap PCR
bold regions indicate added restriction sites (e.g. SalI)

↑ ORDER PLACED w/ BIOSOURCE INTERNATIONAL P.O. 941

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ESTABLISH PCR CONDITIONS FOR PCR AMPLIFYING THE EIA/EIB INTERGENIC REGION.

RXN	PRIMERS	TEMPLATE	COMMENTS
1	96.74.1/2	pCITE-3a	EMCV IRES 519bp
2	96.74.1/2	pCITE-3a	
3	96.74.1/2	Ø	
4	96.74.3/6	CN702	Ad E1A SEQUE 286bp
5	96.74.3/6	CN702	
6	96.74.3/6	Ø	
7	96.74.4/5	CN702	Ad E1B SEQUE 396bp
8	96.74.4/5	CN702	
9	96.74.4/5	Ø	

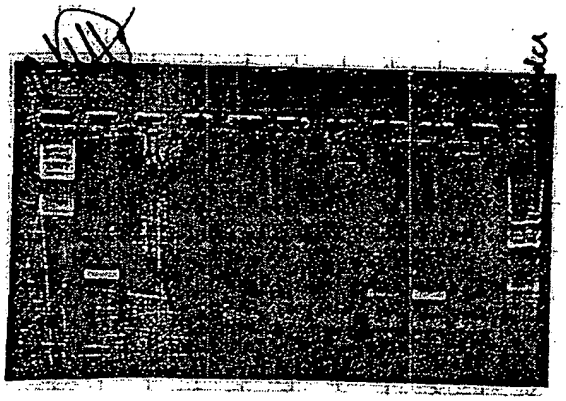
50ul RXN
5ul M PRIMER 1ul
10mM dNTPs ~~5ul~~ 1ul
1ng TEMPLATE 1ul
0.5U DEEP VENT POL 0.25ul

MASTERMIX
50ul BUFF
2.5ul DEEP VENT Po
10ul dNTPs (10mM)
425ul H_2O

94°C X 2'
~~65°C~~
X { 94°C X 45"
65°C X 30" → 55°C 1°/CYCLE
72°C X 30" }

X { 94°C X 45"
55°C X 30"
72°C X 30" }

72°C X 2'
~~4~~ 4°C HOLD



POSITIVE AMPLIFICATION PRODUCT IN LANES 1, 7, 8. AMPLIFICATION OF AD E1A SEQUENCE MUST BE REPEATED. PCR MACHINE UP IS NOT MAKING CONTACT WITH TUBES!

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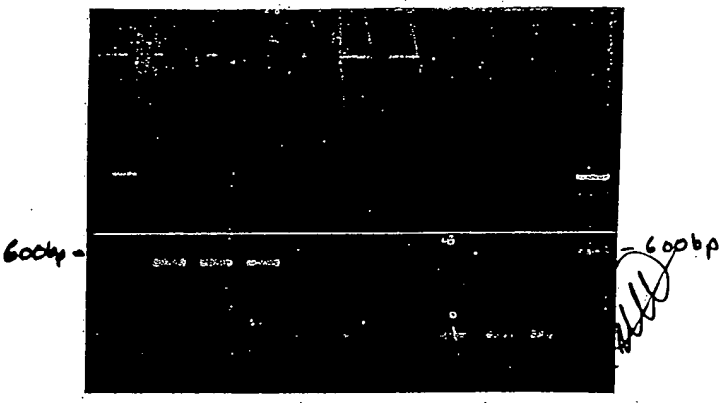
IRES CLONING ~~PLATFORM~~ CONSTRUCTION 96.76

BECAUSE PCR WAS NOT OPTIMAL IN FIRST ATTEM LIKELY BECAUSE LID ON PCR MACHINE IS NOT MAKING GOOD CONTACT WITH TUBES, I WILL REPEA RXNS FOR BETTER YIELD. SAME SETUP AS 96.76



1% AGAROSE 0.5XTAE

EXPECTED EMCV IRES PR AMPLIFIED. EIA/EIB INTERG REGION NOT SUCCESSFULLY AMPLIFIED. I WILL REDO THESE REACTIONS AT 50° ANNEALING TEMP AND US A "HOT START".

1/12/99

REPEAT PCR TO OPTIMIZE CONDITIONS - MODIFY 96.85 BY ALTERING THE ANNEALING TEMPERATURE AND USING A HOT START.

RXN	PRIMERS	TEMPLATE	SIZE	MASTER
1	96.74.3/6	CN702	2886p	96.76
2	96.74.3/6	CN702	~~2886p~~	
3	96.74.3/6	—		LEAVE O Polymer
4	96.74.4/5	CN702	396 bp	
5	96.74.4/5	CN702		
6	96.74.4/5	—		

OLIGOS 96.74.7 THROUGH 96.74.10 WERE RECEIVED FROM BIOSOURCE INTERNATIONAL. TO RECONSTRUCT THE MOUSE VEGF IRES DESCRIBED BY HUEZ (1998) CALLED SP163 OLIGOS 96.74.7 AND 96.74.10 WILL BE ANNEALED AND OLIGOS 96.74.8 AND 96.74.9 WILL BE ANNEALED.

~~50ul~~

OLIGOS WERE REHYDRATED WITH H_2O AT 1ug/ul. 50ul OF 7 AND 10 WERE MIXED AND 50ul OF 8 AND 9 WERE MIXED. BOTH MIXTURES WERE DENATURED @ 100°C FOR 10' AND ALLOWED TO COOL TO ROOM TEMPERATURE TO ACCOMPLISH ANNEALING.

11ul OF T4 DNA LIGASE BUFFER WERE ADDED TO EACH TUBE FOLLOWED BY 1ul NEB PNK (10u/ul) TO PHOSPHORYLATE OLIGOS. INCUBATE @ 37°C X 1H.

HEAT INACTIVATE PNK @ 65°C X 20'

~~SUPPLEMENT RXN WITH~~

Ø-OH/$CHCl_3$ EXTRACT, ETOH ↓, RESUSPEND IN ~~40ul~~ 47ul H_2O ADD 10ul DNA LIGASE BUFFER AND 1ul T4 LIGASE. INCUBATE @ ~~RT~~ RT ~~O/N~~ O/N.

ELECTROPHORESE 3ul OF UNLIGATED AND LIGATED OLIGOS TO CONFIRM SUCCESS OF RXNS.

SOME LIGATION APPEARS TO BE OCCURRING ILLUSTRATED BY THE SMEARING IN LANE 3. I WOULD HAVE EXPECTED BAND @ ~190, HOWEVER. ⇒ CONCATEMERS CAN BE RESOLVED BY SALI DIGEST

To Page No. 89

IRES CLONING - PLATFORM CONSTRUCTION

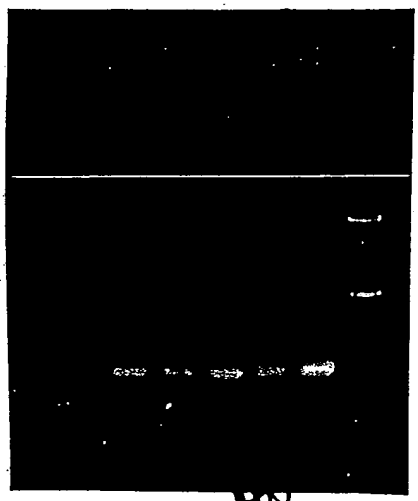
85 96

RXN PROFILE

94°C X 2'
94°C X 45" } 30X
50°C X 30"
72°C X 30"
4°C HOLD

ADD POLYMERASE AFTER 94°C DENATION.

ELECTROPHORESE 1% AGAROSE 0.5X TAE



NO PCR AMPLIFICATION SEEN. I NEED T CHECK PRIMERS TO MAKE SURE THEY ARE CORRECT.

1/13/99 REPEAT PCR USING NEW TEMPLATE DNA (CN510 -70.166.5) AND EXPAND LONG POLYMERASE

MASTERMIX #1 (DEEPVENT)
✓ 7ml dNTPs
✓ 35ml DEEPVENT BUFF.
✓ 298 ml H_2O

MASTERMIX #2 (EXPAND LONG)
7ml dNTPs
35ml EXPAND LONG BUFF.
298 ml H_2O

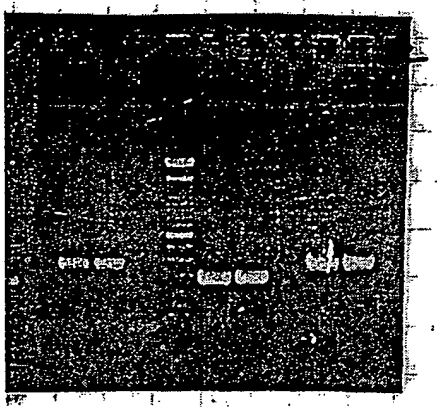
ADD 0.25ml PRIMERS TO EACH RXN
ADD 0.25ml DEEPVENT OR EXPAND LONG POL
ADD 1ml CN510 (1ng) TO EACH RXN, EXCEPT CONTROL

RXN	PRIMERS	TEMPLATE	
1	96.74.3/6	CN510	MASTERMIX #1
2	96.74.3/6	CN510	
3	96.74.3/6	Ø	
4	96.74.4/5	CN510	
5	96.74.4/5	CN510	
6	96.74.4/5	Ø	
7	96.74.3/6	CN510	MASTERMIX #2
8	96.74.3/6	CN510	
9	96.74.3/6	Ø	
10	96.74.4/5	CN510	
11	96.74.4/5	CN510	
12	96.74.4/5	~~CN510~~ Ø	

PCR PROFILE

"HOT START"

94°C x 2'
94°C x 45"
55°C x 30" } ~~30X~~ 25X
72°C x 30"
4°C HOLD



BOTH PRIMER PAIRS GIVE EXPECTED PCR AMPLIFICATION PRODUCT (288 + 396 bp). CHANGING DNA TEMPLATE SEEMS TO HAVE MADE THE DIFFERENCE. I WILL GEL PURIFY PRODUCTS FROM DEEP VENT POLYMERASE BECAUSE OF ITS BETTER FIDELITY THAN TAQ.

sed & Understood by me,

Invented by

Recorded by

IRES CLONING - VEGF IRES 96

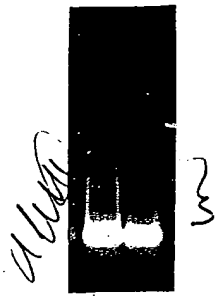
86

1/13/99 AFTER ALLOWING LIGATION TO PROCEED O/N, I Ø-OH/$CHCl_3$ EXTRACTED RXN AND ↓ DNA W/ ETOH.

RESUSPEND DNA IN 75ul H_2O. ADD 10ul BSA ADD 10ul OF 10X SALI BUFFER AND 5ul SALI (50U).

DIGEST O/N @ 37°C

ELECTROPHORESE LIGATED AND CUT SAMPLES TO MONITOR PROGRESS OF RXN.



DIGESTION WITH SAL I SEEMS TO HAVE RESULTED IN LOSS OF HIGHER MOLECULAR WEIGHT DNA CONCATEMERS. BUT I AM CONCERNED SINCE MOST OF THE PRODUCT IS UNLIGATED PRODUCT. I MAY ORDER NEW PNK AND/OR GEL PURIFY OLIGOS BEFORE ATTEMPTING LIGATION.

2/22/99 I AM TURNING THIS PROJECT OVER TO MELISSA SCOTT.

From Page No. 96

~~STRATEGY~~

APPRX 200 COLONIES ON EACH LIGATION PLATE. PICK 6 FROM EACH AND GROW MINIPREPS O/N IN 50ng/ml KANAMYCIN.

PURIFY PLASMID DNA USING FPM KIT AVAILABLE FROM BIO101. ELUTE DNA IN 50ml OF H_2O

DIAGNOSTIC DIGESTS AS FOLLOWS:

pCRBLUNT + PLATFORM 96.91

ACC/NOTI	(+)	37666bp
		4166bp
	(-)	3865bp
		317bp
KBA/KPNI	(+)	3417bp
		601
		91
		73
	(-)	3417bp
		593
		93
		79

pCRBLUNT + EMCV 96.9

HINDIII	(+)	3808bp
		2246bp
	(-)	3627bp
		405bp

LET DIGEST PROCEED O/N @ 37°C

ACC/NOTI
15ml DNA
2ml BUFF4
2ml BSA
1ml ENZYME TOTAL
(5U EACH)

XBAI/KPNI
15ml DNA
2ml BUFF2
2ml H_2O
1ml ENZYME
(5U EACH)

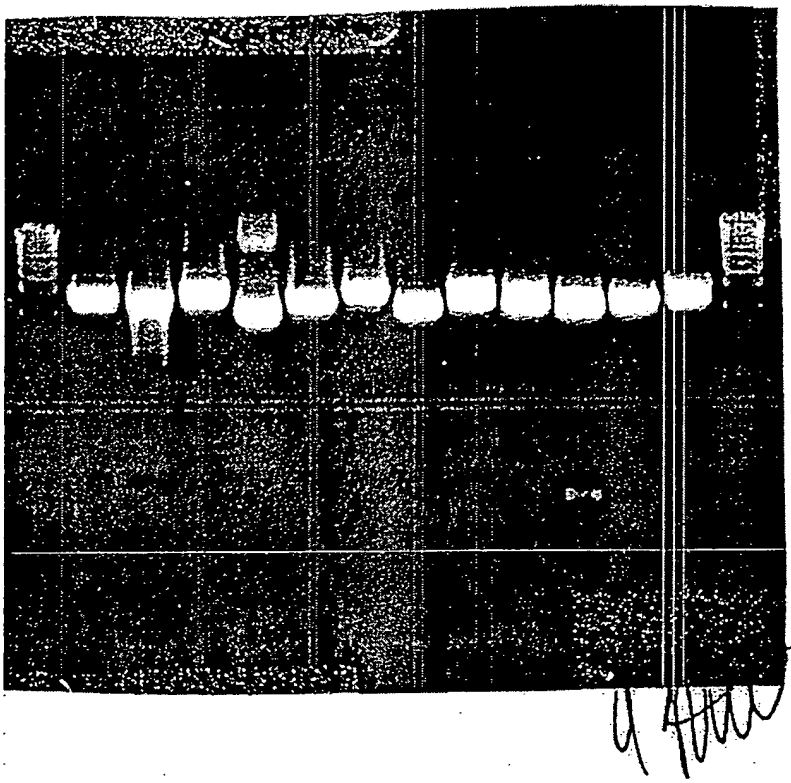
HINDIII
15ml DNA
2ml BUF2
2ml H_2O
1ml ENZYME
(5U)

To Page No.

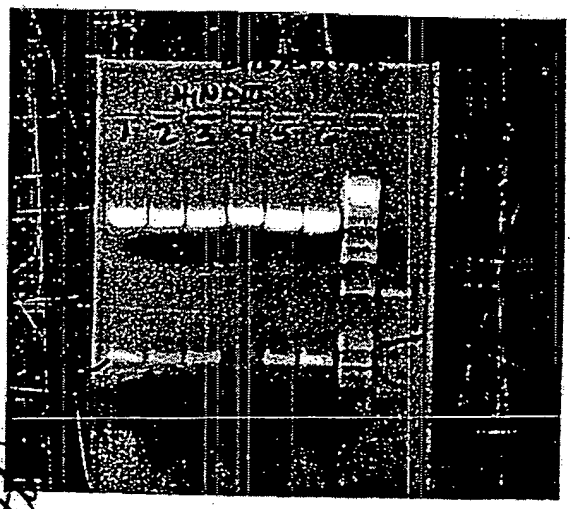
Witnessed & Understood by me,

Invented by

ELECTROPHOREESE 1% AGAROSE



~1.6 kb



ACC/NOTI DIGEST SUGGESTS THAT CLONES 2, 3, 6 ARE RECOMBINANTS IN (-) ORIENTATION. CLONE 1 IS A RECOMBINANT IN THE (+) ORIENTATION. THE XBA/KPNI DIGEST APPEARS TO BE A PARTIAL DIGEST. A 0.6 kb BAND IS EXPECTED, BUT THE 0.7kb BAND IS UNEXPECTED. THE DIFFERING INTENSITIES OF THE BAND SUGGESTS THAT THIS IS A PARTIAL DIGEST ALSO. ANOTHER 0.1kb BAND IS EXPECTED, BUT RAN OFF THE GEL. THE 0.1 kb + 0.6 kb = 0.7kb EXTRA BAND. I WILL GROW UP MAXIS OF CLONES 1, 2 FOR ADDITIONAL CHARACTERIZATION.

CLONES 1, 2, 3, 5, 6 ARE (-) RECOMBINANTS, BUT 4 IS A (+) RECOMBINANT. I WILL GROW UP MAXI OF 1 AND 4 FOR ADDITIONAL CLONING AND CHARACTERIZATION.

Witnessed by me

Date 11. 11/11

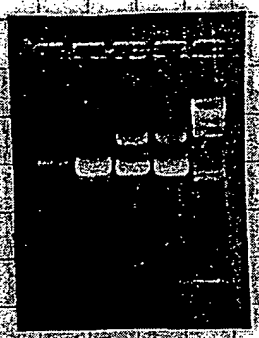
Invented by

rom Pa 100

INOCULATE 200ml CULTURES OF CLONES MENTIONED ON 96.100 WITH LEFTOVER BACTERIAL CULTURES. ##
(KANAMYCIN 50ug/ml). GROW O/N @ 37°C.

PURIFY PLASMID DNA USING QIAGEN MAXI KIT AS SUGGESTED BY THE MANUFACTURER. ↓ DNA W/ ISOPROPANO RESUSPEND IN 500ul H_2O.

ELECTROPHORESE 1ul OF EACH PREP TO DETERMINE CONCENTRATION.



CONCENTRATION OF 96.96 1 #1 = 5ng/ul
96.96 1 #2 = 200ng/ul
96.96 2 #1 = 200ng/
96.96 2 #4 = 200ng/

DIAGNOSTIC DIGESTS TO RECONFIRM STRUCTURE
PREP DIGESTS FOR CONTINUING CLONING

KPNI/XBAI

✓5ul CLONES 96.96 1 #1 AND #2
✓2ul BUFF 2
10 UNITS XBAI/KPNI
✓12ul H_2O
20ul TOTAL

SALI

✓5Dul CLONE 96.96 1
✓20ul SALI BUFF
4ul SALI (50U)
20ul BSA
✓106ul H_2O
100ul TOTAL

Witnessed & Understood by me,

Invented by

Page No.

DIGEST STRATEGY

HINDIII

✓5ul DNA CLONES 96.96 2 #1/#4
✓2ul BUFF #2
✓1ul HINDIII (5U)
✓12ul H_2O

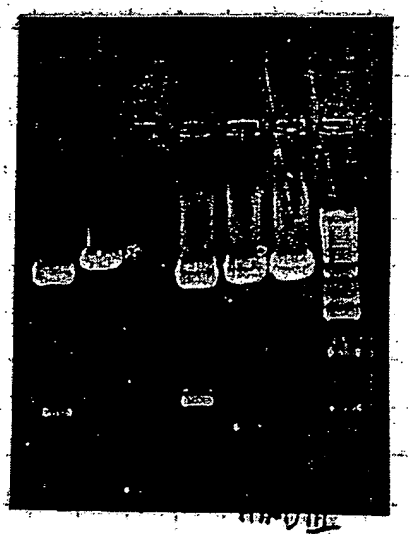
20ul TOTAL

SALI

50ul DNA CLONE 96.96
20ul SALI BUF #1
20ul BSA
4ul SALI (80U)
106ul H_2O

200ul TOTAL

ELECTROPHOREESE 5ul OF PREP DIGESTS AND 15ul OF 29 DIAGNOSTIC DIGESTS TO MONITOR RXNS. 1% AGAROSE.



DIGESTS YIELD EXPECTED RESULTS AS INDICATED ON PHOTOGRAPH SALI DIGESTS OF VECTOR (96.96.1 #2) AND INSERT (96.96.2 #1) WERE SUCCESSFULL. NEXT STEP IS TO CIP VECTOR AND THEN GEL PURIF BOTH VECTOR + INSERT PRIOR TO LIGATION.

96.96.1 #2 = CP 624
96.96.2 #1 = CP 625

Ø-OH/ $CHCl_3$ EXTRACT VECTOR, PRECIPITATE W/ 3 VOLUMES ETOH. RESUSPEND PELLET IN 90ul H_2O. ADD 10ul OF NEB BUFFER 3. ADD 2ul CIP. INCUBATE RXN FOR 1hr @ 37°C. STORE @ -20°C UNTIL PURIFICATION.

essed & Understood by me.

Invented by

Recorded by

TITLE PLATFORM PLASMID + EMCV IRES CLONING

Project No.

Book No. 96

From Page No. 102

GEL PURIFY INSERT AND VECTOR – 1% AGA

EXTRACT BANDS, PURIFY DNA
USING QIAGEN QIAEXII KIT
ELUTE W/ 10ul H_2O.

ELECTROPHORESE 1ul 0.8%
AGAROSE TO DETERMINE YIEL

[VECTOR] = 20ng/ul
[INSERT] = 5ng/ul

LIGATION SETUP

A	B	C	
✓2ul VECTOR	✓2ul VECTOR	✓2ul INSERT	
✓2ul INSERT	1ul LIGASE	1ul LIGASE	16°C O/N
1ul LIGASE	1ul BUFF	1ul BUFF	
1ul BUFF	6ul H_2O	6ul H_2O	
4ul H_2O			

DILUTE LIGATIONS 1:10 IN H_2O. TRANSFORM DH10B WITH 1ml BY ELECTROPORATION. ALLOW TO RECOVER FOR 45' @ 37°C. PLATE ON KAN PLATES INCUBATE @ 37°C O/N.

TMC COLONIES ON PLATE A AND B. DIFFICULT TO DETERMINE IF THERE IS ANY DIFFERENTIAL
NO COLONIES ON PLATE C.
PICK 12 COLONIES FROM PLATE A. GROW O/N IN KAN/LB.

PURIFY PLASMID DNA FROM MINICULTURES USING BIO 101 RPM KIT (LOT # 2070-600-536-3).
ELUTE DNA w/ 50ml H_2O.

DIAGNOSTIC DIGESTS w/ HINDIII TO DETERMINE RECOMBINANTS.

EXPECTED DIGESTS:

PLATFORM + IRES (+) ORIENTATION	3.9kb
	0.8kb
PLATFORM + IRES (-)	4.1kb
	0.6kb

*N
SETUP → 15ml DNA
2ml NEB 2
3 UNITS HINDIII
3ml H_2O

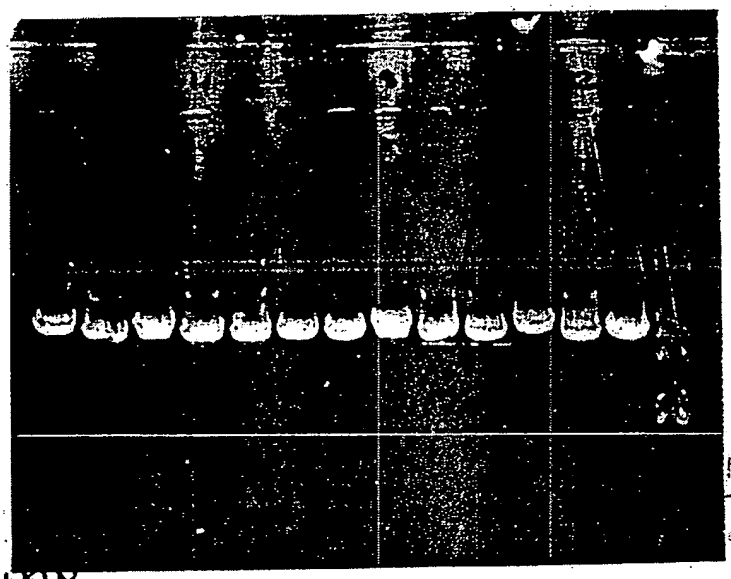
DIGEST FOR 5hrs @ 37°C

To Page No. 105

PLATFORM-IRES CLONING 104

104

ELECTROPHORESE SAMPLES – 0.6% AGAROSE GEL 90V
0.5XTAE

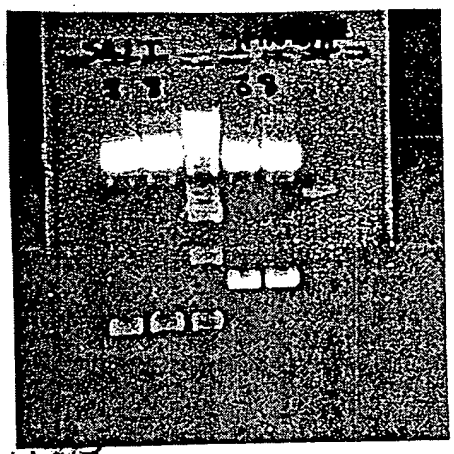


CLONES 3 AND 8 AR
RECOMBINANTS. HOW
SINCE RESOLUTION IS
POOR, I CANNOT DETER
MINE ORIENTATION;
I WILL REPEAT THIS
DIGEST TO CONFIRM
CLONES.

VECTOR CONTROL
YIELDED LINEARIZED
PRODUCT ~4kb AS
EXPECTED.

REDIGEST CLONES 3
8 WITH HINDIII (4KE
AND SALI (96.101) 5h
@ 3:

ELECTROPHORESE – 1%
AGAROSE, 0.5X TAE



SAL I DIGEST, 3 + 8 ARE AS
EXPECTED (INSERT @ 0.5kb). HINDI
DIGEST IS CONSISTENT W/ (+)
ORIENTATION RECOMBINANT. I WIL
STREAK 3 + 8 ON PLATE
AND GROW MAXI PREPS PRIOR
TO SEQUENCING.

96.103.3 = CP626

IRES CLONING → CN306 96

105

2 200ml CULTURES INOCULATED (50µg/ml KAN). GROW O/N @ 37°C

PURIFY 96.103.3 AND 96.103.8 DNA USING QIAGEN MAKIPREP KIT. ELUTE DNA IN 500µl H_2O. STORE @ -20°C UNTIL NEEDED.

BEGIN CLONING OF PLATFORM PLASMID CONTAING THE EMCV IRES INTO CN306 BACKBONE

DIGEST 96.103.3 AND CN306 (96.98) WITH KPNI AND XBAI.

96.103.3 (GPG26)
✓50µl DNA (96.107) 10µg
✓20µl BUFF #2
4µl XBA (80U)
✓4µl KPNI (80U)
✓122µl H_2O

CN306
✓50µl DNA (96.98) -1
✓20µl BUFF #2
4µl XBA (80U)
✓4µl KPNI (80U)
✓122µl H_2O

DIGEST O/N @ 37°C

ELECTROPHORESE 10 ul OF EACH RXN TO MONIT PROGRESS OF RXN.

CN306 DIGEST IS COMPLETE. HOWEVER, 96.103.3 DIGEST PRODUCED UNEXPECTED RESULTS. AFTER INSPECTION OF VIRTUAL CONSTRUCT, I REALIZED THAT 96.103.3 HAS A KPN1 SITE IN THE IRES THAT I HAD NOT ACCOUNTED FOR. EXPECTED BANDS ARE, THEREFOR,

3.4 kb
0.6
0.47
0.09
0.07

RESULTS ARE CONSITENT.

TO GET AROUND THIS PROBLEM, I WILL DO A PARTIAL KPN1 DIGEST AND GEL PURIFY 1kb BAND.

DIGEST 96.103.3 WITH XBA1 COMPLETELY. FOLLOW UP WITH PARTIAL KPN1 DIGEST TOMORROW.

130 ul DNA (26 ug)
20 ul BSA
20 ul BUFF 2
6 ul XBA1 (120 UNITS)
24 ul H_2O

DIGEST O/N @ 37°C

To Page No. 10

essed & Understood by me,

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Invented by

Recorded by

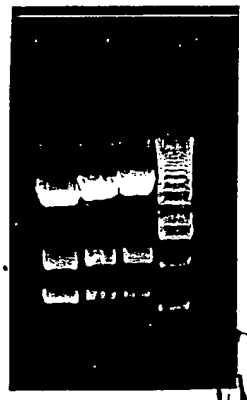
IRES CLONING 96

108

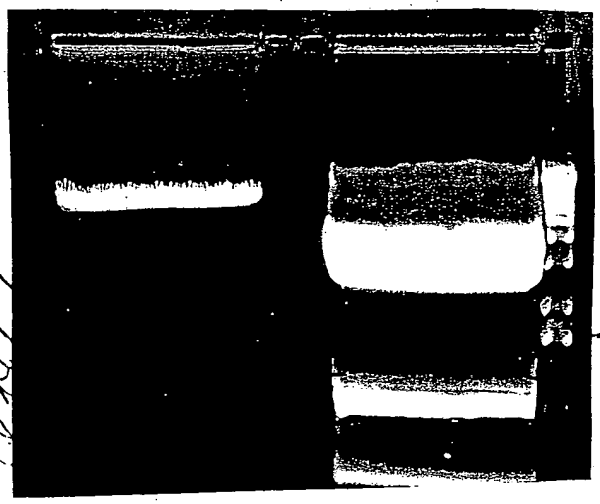
DIVIDE XBAI DIGEST INTO 3, 65ul/TUB DIGEST EACH TUBE FOR 1H @ 37°C AS BELOW:

#1	#2	#3
20 UNITS KPN I	10 UNITS	5 UNITS

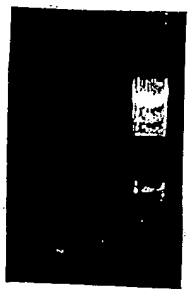
AFTER RXN INCUBATION, PLACE TUBES ON ICE ELECTROPHORESE 5ul TO MONITOR RXN PROGRESS.



PARTIAL DIGEST WAS SUCCESFULL. ~1kb BAND IS PRESENT. NEXT STEP IS TO PURIFY BAND BY ELECTROPHORESIS



PURIFY DI
BY QIAERI
KIT- ELUT
W/ 10ul H



DETERMINE [DNA] PRIOR TO LIGATION

[96.103.3] = 20 ng/ul

~~[TASL~~

[VECTOR] = 10ng/ul

11

Andrew S Ellington

IRES + CN306 LIGATION 96

109

LIGATION

A	B	C
✓ 5ul VECTOR (50ng/ul)	✓ 1ul IRES INSERT	✓ 5ul VECTOR
✓ 1ul IRES INSERT	✓ 1ul BUFF	✓ 1ul BUFF
✓ 1ul BUFF	✓ 1ul LIGASE	✓ 1ul LIGASE
✓ 1ul LIGASE	✓ 7ul H_2O	✓ 3ul H_2O
✓ 2ul H_2O		

INCUBATE RT X 2HR

TRANSFORM LIGATIONS (DILUTED 1:10) DH10B ECETRO COMPETENT CELLS. PLATE ON LB/AMP PLATES. INCUBATE O/N.

TMC COLONIES ON PLATES A AND C. PICK 10 FROM PLATE A FOR MINILYSATES. GROW O/N @ 37°C.

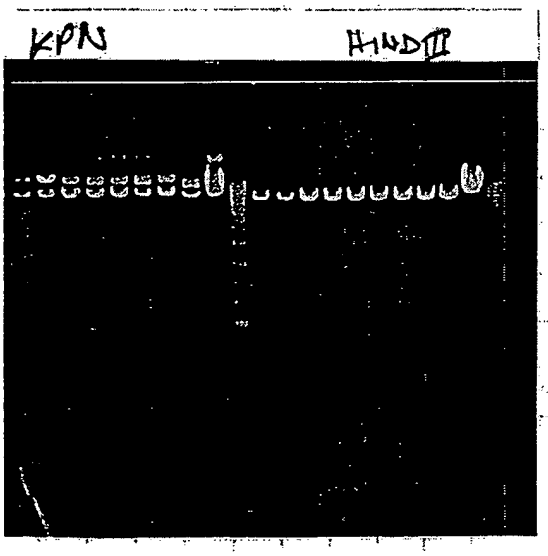
PURIFY PLASMID DNA USING RPM BIO101 KIT. ELUTE PLASMID DNA IN 50 ul OF H_2O. CHARACTERIZE RECOMBINANTS WITH KPNI AND HINDIII DIGESTS.

DIGEST SETUP: SCREEN FOR RECOMBINANTS.

KPNI	HINDIII
15 ul DNA	15 u DNA
2 ul BSA	2 ul BUF 2
2 ul BUFF 2	1 ul enzyme (34)
1 ul enzyme (34)	2 ul H_2O

DIGEST 4hrs @ 37°C

ELECTROPHORESE @ 90V, 0.8% AGAROSE 1hr



EXPECTED BANDS:

HINDIII – 8.3kb
1.4 kb

KPNI – 9.3kb
0.5kb

CN306 VECTOR SHOULD BE LINEARIZED IN EACH CASE

KPN1 DIGEST DOES NOT APPEAR COMPLETE. HOWEVER, HIND3 DIGEST INDICATES THAT CLONES 96.115 1, 2, 3, 4, 5, 6, MAY BE RECOMBINANTS. ≠ BAND @ 1.5kb IS INDICATIVE. CLONES 1 AND WILL BE MAXIPREPED, AND THEIR STRUCTURE WILL BE CONFIRMED WITH ADDITIONAL DIGESTS BEFORE CONTINUING.

NOTE: THIS CLONING IS BEING TURNED OVER TO MELISSA SCOTT

essed & Understood by me.

Invented by Andrew S

Recorded by

Proj. No.

TITLE EMCV IRES CLONING

Book No. 96

From Page No. 116

INOCULATE 250ml LB/AMP WITH BATERIA FROM 96.11
AND 96.115.2. GROW O/N @ 37°C SHAKING.

PURIFY PLASMID DNA USING QIAGEN MAXIPREP KIT
AS SUGGESTED BY MANUFACTURER. RESUSPEND
DNA IN 500ul H2O

CLONING TURNED OVER TO MELISSA SCOTT.

Witnessed & Understood by me,

Invented by

Project No
Book No. 19 TITLE IRES Strategy

From Page No.__

GOAL → to regulate the expression of both the E1A gene and the E gene by using a single tissue specific promoter. To do so an internal ribosome entry site (IRES) must be cloned b/t the 2 g
↳ an internal entry point for initiation of translation by euk. ribosomes

2 Potential IRES:

① EMCV: encephelomycarditis virus. Well characterized. Commercially available from Novagen. (450bp) efficient translation. dicistronic message, commonly used in gene therapy.

② VEG-F: from mouse, vascular endothelial growth factor. Active in hypoxic environments, 1000nt in length

Advantages of including IRESes In ARCA system
- regulate expression of two Ad genes from a single tissue specif
- regulate expression of one Ad gene and one cytotoxic gene (CD, P450, IL4) from a single TRE
- prevent homologous recombination
- preferential expression of IRES controlled gene in hypoxic cel

Want to design a platform plasmid for constructing Ad mutar that contain IRESes

Picking up project in the beginning → where Andy left off.

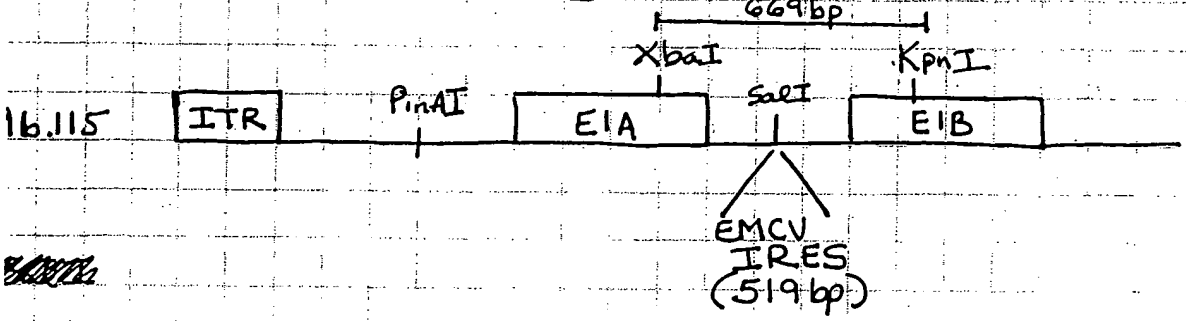
Witnessed & Understood by me,
Heather Connors

Invented by

Page No.

rom Page No. 114

Andy created the following deletion in CN306 (derived from pXC.
↳ lacks the E1a promoter alrea
Deleted the E1B promoter and E1a poly a signal, but leaves the E1a + E1b coding regions intact.
Final Construct looks like this:



used overlappi
PCR to delete
E1B promoter
poly a signal
at the same ti
introduced an
SalI site for
insertion of
IRESes

Andy → the EMCV IRES was amplified from pCITE3 (Novagen) w/ SalI ends that were used for ligation into the platform plasmid.

The PCR produ
was then cloned
back into CN3
↑XbA/KpnI

The IRES insert will be 6 bases upstream of the E1B translation initiation codon.

The EMCV IRES contains a pyrimidine rich tract of bases near its 3' end that are imp. for its activity. This pyrimidine tract will be 27 bases from the E1b initiation codon.

This is where Andy is at. He would like me to confirm the [redacted] clone which is the platform plasmid + EMCV IRES. This clone is called 96.115 for now.

Once clone is confirmed WANT TO ADD 2 different promoters:

① PB: probasin promoter, prostate. Can obtain from CP251 w/ [redacted] Pi.

② CMV: cytomegalovirus promoter, expressed 100 fold more. Need to amplify w/ PinAI sites 5' + 3'

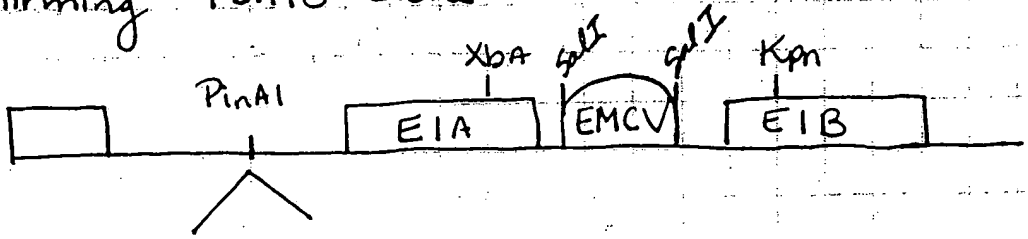
CV787 has prostate specific promoter. CMV active in all cells. (100 fold more) Very Activ

To Page No. 11

Witnessed & Understood by me,

Invented by

onfirming 96.115 clone



① PB promoter
② CMV promoter

CN251 ↑PinAI fragment
design primers for PCR ↑PinAI 5' and 3'

1. Digest : Andy has 2 potential clones that need to be confirme

4 Sets of Digests:

Xba/KpnI buffer 2 +BSA

CP306: 8600 bp, 700 bp
96.115 #1+#2: 8656 bp, 641, 467

KpnI buffer 1 +BSA

CP306
96.115 #1 + #2

Cla/AvrII buffer 4 + BSA

CP306 9.4 Kb, linear no AvrII
96.115 #1+#2

PinAI Gibco buffer 4. Doing this digest now in case clones are correct then can proceed right away w/ ligating PB promoter into platf construct.
CP251 DNA from DC 1.15 µg/µl (used 2µl for digestion)

To Page No. 11

Witnessed & Understood by me,
Heather Connors

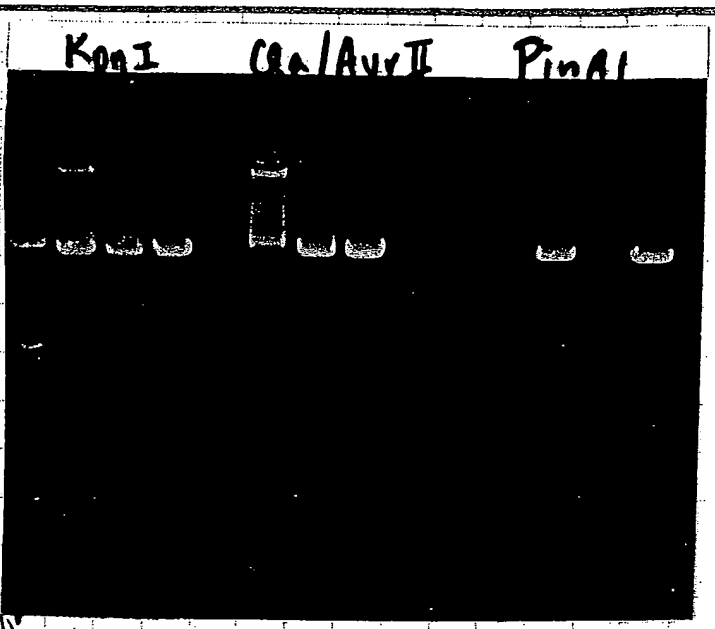
Invented by

Recorded by Melissa Smith

TITLE _____ Proj No. _____ Book No. 99

From Page No 116

Xba/Kpn



Results

Xba/Kpn: CP 306 dropped the correct size fragment → 700 bp
9b.115 # 2 is correct. Think #1 is correct too – just difficult to see BANDS in the ~~gel~~ dye BAND.

Cla/AvrII: ClaI is not cutting due to dam methylation!! Overlooked this. ∴ CP306 is uncut. #1 + #2 are linearized by AvrII

PinAI: #1 + #2 look OK. CP25~~6~~1 is not 1.15 µg/µl!

Next: believe # 2 looks correct. WANT TO be sure.
Use more DNA + rpt digests: ~~o~~ Digest all 3 constr.

① Xba/KpnI buffer 2 + BSA; 10µl DNA

② Hind III 10µl DNA
looking for 1.4 and 8.3 Kb band.

CP251: inoculated o/N culture from glycerol stock LB+

Witnessed & Understood by me,

Invented by

rom Page No. 11

	Xba/KpnI	H3	DNA [ ]
CP306	buffer 2	buffer	10μl (~2μg)
96.115 #1	+	2	10μl (~2μg)
96.115 #2	BSA		

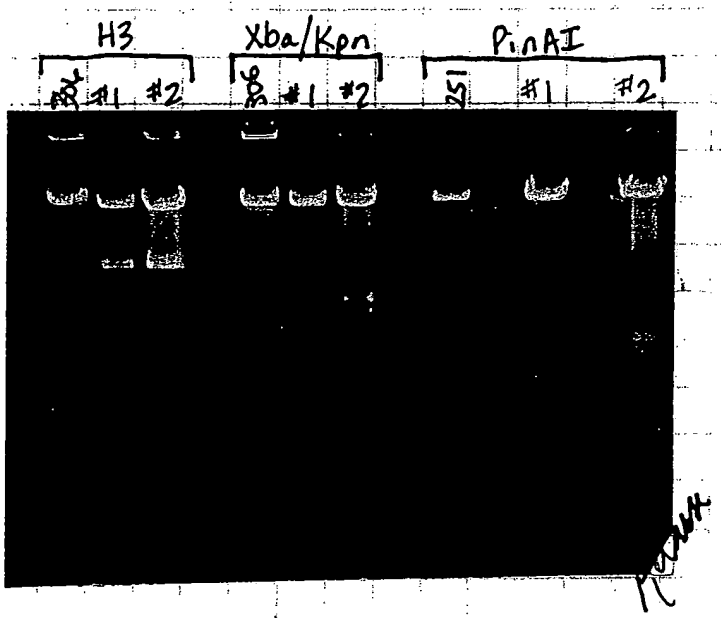
@ 37°C 9AM –

Also repeated PinAI digest.
2 mini-preps of CP251. Will add 1 tube to company DNA stock. (~20
Digested the following samples w/ PinAI:

CP251 25μl DNA (~2.5μg)
3μl Gibco buffer 4
2μl PinAI (10 units)
30μl

96.115 #1+2 20μl (~4.0μg)
5μl H_2O
3μl Gibco buffer 4
2μl PinAI
30μl

@ 37°C 9:30–



Longer Run

Results: MARKER looks terrible. Try using Andy's 1Kb ladder.
HARD to tell size of H3 fragments. Need to rpt digest! (aga
Xba/Kpn → fragments so faint but both look OK.
PinAI: Andy thinks maybe there is not enough DNA for 251
so 500 bp fragment isn't showing up.

To Page No. 11

Witnessed & Understood by me, Heather Connors

Date

Invented by

Recorded by Melissa Scott

From Page No. 118

Next: Do maxi-prep for 251 Final Volume 400µl

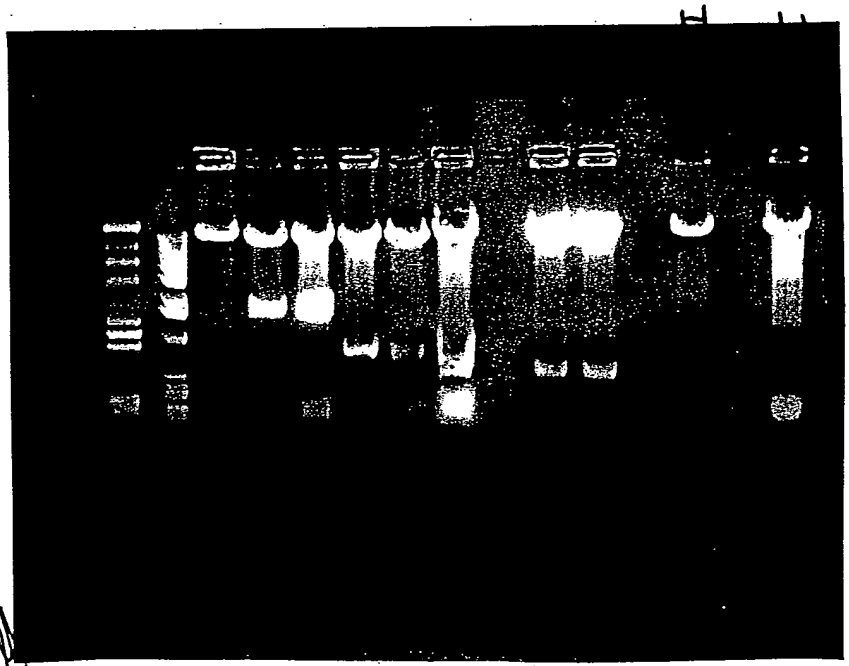
Then set up the following o/N digestions: (maybe digestion will be more complete)

↑H3 buffer 2: 10µl DNA CP306, 96.115 #1 + #2

↑Xba/Kpn buf2+BSA: 10µl DNA CP306, 96.115 #1 + #2

PinAI: 96.115 #1 + #2: 25µl DNA
3µl buffer
2µl enzyme
30µl

CP251: 100µl DNA (~20µg)
12µl buffer
8µl PinAI (40 Units)
120µl



96.115 #1 + #2 look correct

Purified: CP251 500bp fr
96.115 ↑PinAI #1 9Kb
#2 9Kb

CIP Treated Both #1 and #2
(used same protocol as on p. 35.
17µl DNA (gel purified)
2µl buffer
1µl CIP (Boehringer)
20µl
@37°C 1h

Added 0.2µl of .5M EDTA
@75°C 10 min
Phenol/$CHCl_3$
Etoh ppt (15µl)
Resuspend in 15µl H_2O

CP627: 96.115 #2

★See page 135

To Page N

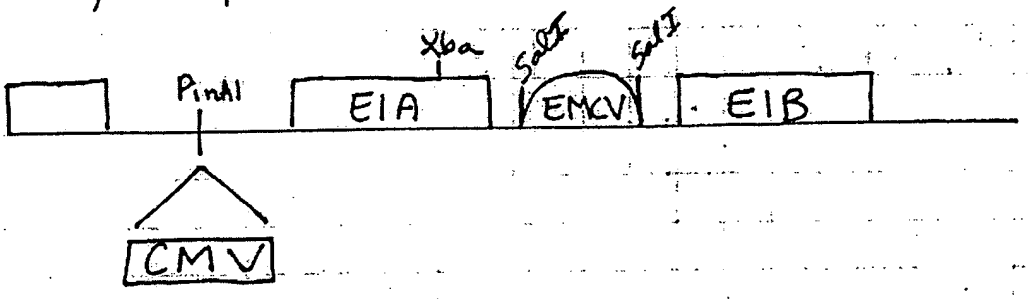
Witnessed & Understood by me,

Invented by

Recorded

GOAL → also want to add CMV promoter to platform construct... PCR promoter + clone into PinAI site of 96.115

CMV promoter will act as a control → the probasin promoter is specific to the prostate. CMV is active in many different types of cells. Can use these 2 for comparison → look at specificity of probasin construct.



Primer Design

2/23/99

99.120.1 FW Primer PinAI (Anneals to 27-41 in pCMVB, Clontech) Tm= 40C

5'- ACG TAC ACC GGT CGT TAC ATA ACT TAC -3'

99.120.2 RV Primer PinAI (Anneals to 535-548 in pCMVB, Clontech) Tm= 42C

5'- CTA GCA ACC GGT CGG TTC ACT AAA CG-3'

Witnessed & Understood by me,

Heather Connors

Invented by

Recorded by

Proje... No.
TITLE Ligation of PB into Platform Construct Book No. 99

From Page No 119

Ligation Platform Construct + Probasin promoter
4µl vector (CIP treated 96.115)
6µl fragment (CP251↑ PinAI)
1.2µl buffer
.8µl ligase
2.0µl
@16°C o/N

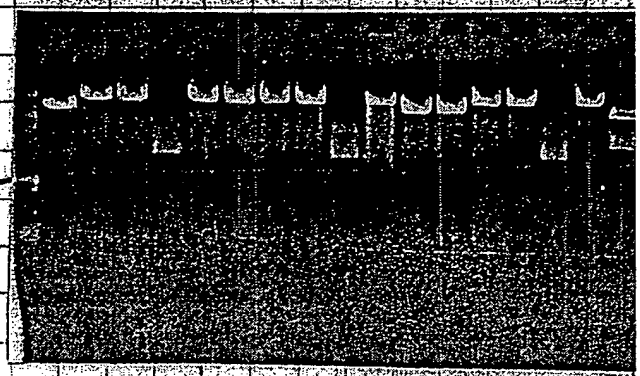
Transform JM109 180µl + 12µl ligation rxn LB+Amp

Colonies w/ both constructs
Stored plates at 4°C → pick colonies 2/28/99

Picked 16 colonies (96.115 #2 + PB promoter)
4ml LB+Amp @37°C o/N

Mini-Preps
R. Digest NruI / BclI Expected fragment size: 6334,
↳ unique in PB promoter

10µl DNA
2µl buffer 7 Gibco
1µl NruI
6µl H_2O
19µl @37°C 1hr
heat inactivate @65°C 20min
add 1µl BclI @50°C 1hr



Ran gel longer → see page 129

BEST AVAILABLE COPY

Witnessed & Understood by me.

Invented by

10 Transformation very efficient — maybe self-ligation.
Check 9 transformants for each clone.

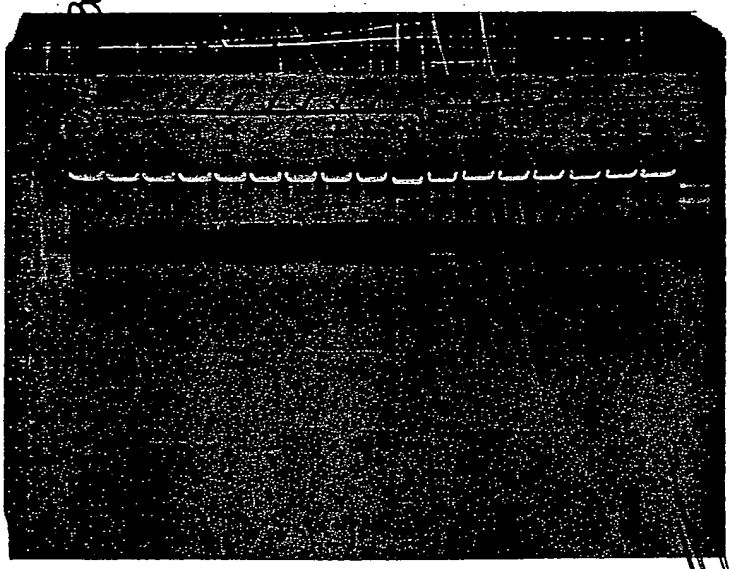
Inoculated 4ml LB + Amp @ 37°C O/N
① #1-9 CMV + CP306
② #10-18 PB + CP306

1 Mini-preps
R. Digest ↑ PinAI buffer 4 Gibco

20µl DNA
2.5µl Gibco 4
2.5µl PinAI (12.5U)
25.0µl

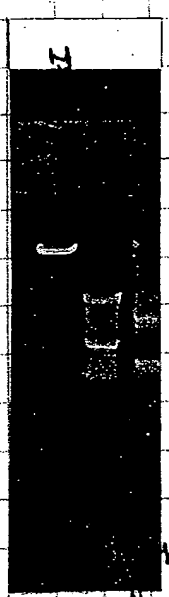
@ 37°C 10:30 - 1:30pm

#18 did not fit on gel!



Results:

CMV 8/9 correct
PB 6/8 correct



tnessed & Understood by me,
Heather Connors

Invented by
Recorded

rm Orientation of the following clones:

~~CP~~ + CMV # 1, 4, 5, 7, 17

~~MT~~ + PB # 2, 3, 5, 6, 7, 8, 13, 14, 16

same digests as before for screening: (p 130)

CMV Constructs: Sst / H3 buffer 2

PB Constructs: ~~Xba~~ / H3 buffer 2 + ~~Eco RI~~

NS NS

@37 ~~10AM~~ - 4pm

15µl DNA
1µl enzyme
1µl enzyme
2µl buffer
20µl

↑Sst / H3 buffer 2

ROL 96.115	Negative Orientation	Positive
5480	4290	4780
2003	2003	2003
1030	1871	1228
841	1718	1030
410	410	841
		410

↑H3 buffer 2

gative Orientation	Positive Orientation	CONTROL
7603	7155	8362
1440	1677	1440
1219	1440	

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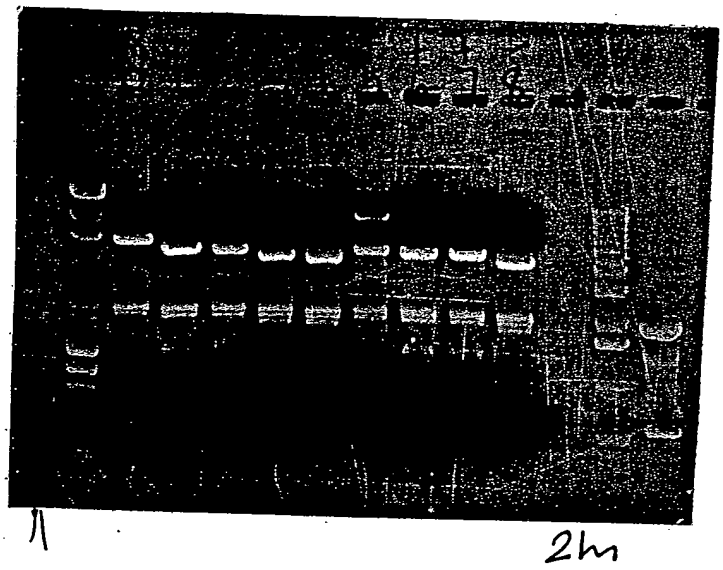
| | |

TITLE CP 632 , CP 633

Project No.

Book No.

From Page No.



Results: 5/8 CORRECT orientat

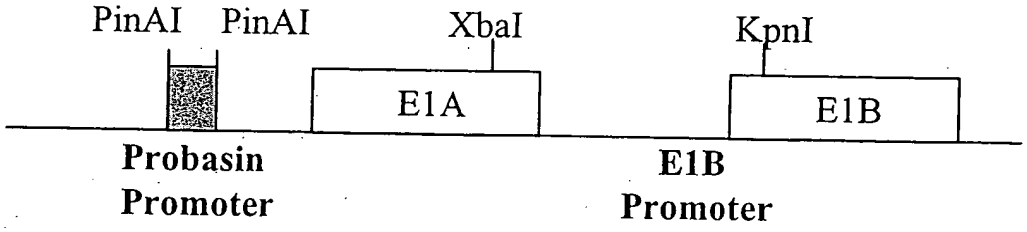
#1, 2, 5, 6, 7

CP633

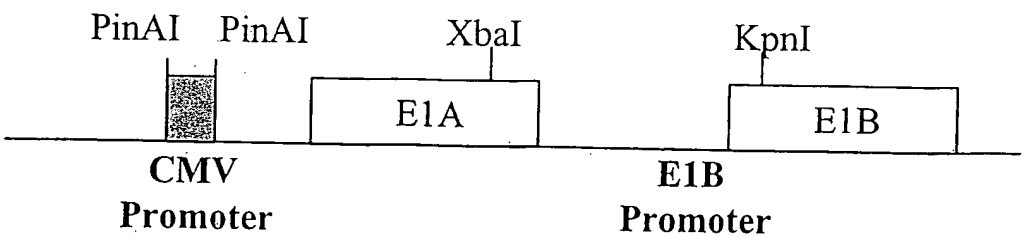
Control Constructs

CP632: #10 99.137 CP306 + Probasin promoter obtained from CP251 digested with PinAI. Control Protein- **E1B promoter** and E1A poly A signal present, no IRES.

Glycer Stocks Prepa 3/16



CP633: #1 99.139 CP306 + CMV promoter amplified from pCMVbeta (Clontech) with PinAI 5' and 3'ends for ligation.Control Protein- **E1B promoter** and E1A poly A signal present, no IRES.



Witnessed & Understood by me. | Date | Invented by

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